

DISAPPEARANCE RATES AND IMMUNOSUPPRESSION OF INTERMITTENT INTRAVENOUSLY ADMINISTERED PREDNISOLONE IN RABBITS AND HUMAN BEINGS

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THE IMMUNOSUPPRESSIVE REGIMENS presently in clinical use for organ transplantation consist of multiple agents, the most widely used combinations being azathioprine and prednisone (15) or azathioprine, prednisone, and heterologous antilymphocyte globulin (16). It is generally conceded that the steroids play an indispensable role in the foregoing therapeutic programs but at the cost of major toxicity in the event of daily high dose requirements for long periods (16).

For more than five years in our institution, an attempt has been made in selected patients to reduce the side effects of prednisone by giving reduced daily doses supplemented by large intermittent injections of intravenously administered prednisolone or methylprednisolone. The indications for such an approach have included refractory rejections as well as infectious complications. Under these circumstances, large intermittent injections of as much as 1 gram of prednisolone have been given every three to ten days.

In view of the empiric nature of this practice and because such large doses of prednisolone have not been employed before to our knowledge, this study was undertaken to determine the duration of the elevated steroid blood levels in human beings after an injection, the degree and persistence

of the consequent lymphopenia, and the immunosuppressive effect of this kind of treatment in human beings and in rabbits.

METHODS

Animal Studies

Full thickness skin grafts, 1 centimeter in diameter, were exchanged by the method of Medawar between 16 New Zealand and 16 California rabbits which weighed from 1.7 to 3.9 kilograms. The skin grafts were placed on the lateral side of the chest, and appropriate autograft controls were performed. Homograft survival was assessed by both visual and microscopic examination, with rejection being declared on the basis of total necrosis of the homograft.

Eight rabbits, four of each breed, were placed in four experimental groups of which the first received no immunosuppressive treatment. A second group of rabbits was given 0.1 milligram per kilogram of methylprednisolone sodium succinate (Solu-Medrol®) intramuscularly each day. A third group was treated with 0.25 milligram per kilogram per day of intramuscularly administered methylprednisolone. The fourth group of rabbits received a combination of the low dose of 0.1 milligram per kilogram per day of intramuscularly administered methylprednisolone plus a high intravenous dose, 10 milligrams per kilogram, every six days beginning on the day of grafting. The therapy with intramuscularly administered methylprednisolone was always begun four days prior to the skin transplantation. The

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TABLE I. DELAYED HYPERSENSITIVITY SKIN TESTS IN HEALTHY VOLUNTEERS WHO RECEIVED 1 GRAM OF PREDNISOLONE TWO HOURS BEFORE THE FIRST ANTIGEN INJECTION

Antigen injection	<i>Mumps</i>			<i>Candida</i>			<i>Vaccinia</i>			<i>Tuberculin</i>		
	Read after			Read after			Read after			Read after		
	1 day	2 days	3 days	1 day	2 days	3 days	1 day	2 days	3 days	1 day	2 days	3 days
1 1st	0	0	0	0	0	0	+3	+3	—			
Control	+1	+1	+1	0	0	0	—	—	—			
2 1st	0	0	0	0	0	0	+1	+3	+3			
Control	+3	+2	—	+2	+3	—	—	—	—			
3 1st	0	+1	+1	0	0	0	0	+2	+2			
2nd, 24 hrs.	+1	+3	—	0	±	±	+2	+3	—			
Control	+2	+3	—	+2	+2	—	—	—	—			
4 1st	0	+2	+4	0	0	0	+1	+4	—	0	0	0
2nd, 48 hrs.	+4	—	—	+1	+2	—	—	—	—	+1	—	—
Control	+3	+3	+3	+2	+2	+2	—	—	—	+1	—	—
5 1st	+1	+3	±	0	±	±	+1	+4	+4	0	+1	+1
2nd, 24 hrs.	+2	+4	—	+1	+3	+1	+1	+1	+1	+1	+1	+1
3rd, 48 hrs.	+2	+3	—	+1	+3	+3	—	—	—	+1	+2	+3
Control	+4	—	—	+3	—	—	—	—	—	+3	—	—
+1 =	1-1.5 cm. erythema and 2-3 mm. nodule			1.5 cm. erythema and 5 mm. nodule			4 mm. nodule			5 mm. nodule		
+2 =	2-2.5 cm. erythema and 3-4 mm. nodule			2-3 cm. erythema and 7 mm. nodule			6 mm. nodule			6-7 mm. nodule		
+3 =	3-3.5 cm. erythema and 4-5 mm. nodule			3-4 cm. erythema and 9 mm. nodule			8 mm. nodule			8-9 mm. nodule		
+4 =	> 4 cm. erythema and > 5 mm. nodule			> 4 cm. erythema and > 10 mm. nodule			> 8 mm. nodule with necrosis			> 10 mm. nodule		

powder form of methylprednisolone was freshly dissolved in the diluent for each day's injections.

The intramuscular dose of 0.1 milligram per kilogram of methylprednisolone corresponded to 0.12 milligram of parenterally administered prednisolone and to 0.18 milligram of orally administered prednisone, which would be a total dose of 12.5 milligrams of orally administered prednisone for a man weighing 70 kilograms. The intramuscular dose of 0.25 milligram per kilogram of methylprednisolone was equivalent to a total of 32 milligrams of prednisone administered orally to a man weighing 70 kilograms. The intravenous dose of 10 milligrams of methylprednisolone per kilogram in the rabbit was comparable to a total of 1 gram of prednisolone in a human being.

Human Studies

Ten patients were studied for one to three days after the intravenous infusion, over 60 minutes, of 1 gram of prednisolone in 50 milliliters of 5 per cent glucose in water. Five of the ten patients had well functioning renal homografts but were imperiled because of wound infections (Fig. 1) or pneu-

monitis. The other five patients were essentially anephric prior to transplantation or had renal homografts that had failed. Eight of the ten patients were receiving concomitant immunosuppressive treatment with azathioprine, and seven of these eight were also being treated with heterologous anti-lymphocyte globulin. The other two patients who were in the pretransplantation period were being given only prednisolone.

In addition, six healthy persons, including some of the authors, volunteered to receive 1 gram of prednisolone by the same infusion technique. Blood samples were taken from these volunteers and from the ten patients before, at the midpoint, and just after completion of the infusion. Additional specimens were taken one, two, four, eight, 12, 16, 24, and 48 hours later. In a few instances, samples were also drawn after three days. When renal failure was not present, the subjects were hydrated from two hours before to two hours after the administration of prednisolone, and inulin and hippuran clearances were determined as described elsewhere by Popovtzer. Urine samples were collected before the prednisolone injection and between the times of blood drawing.

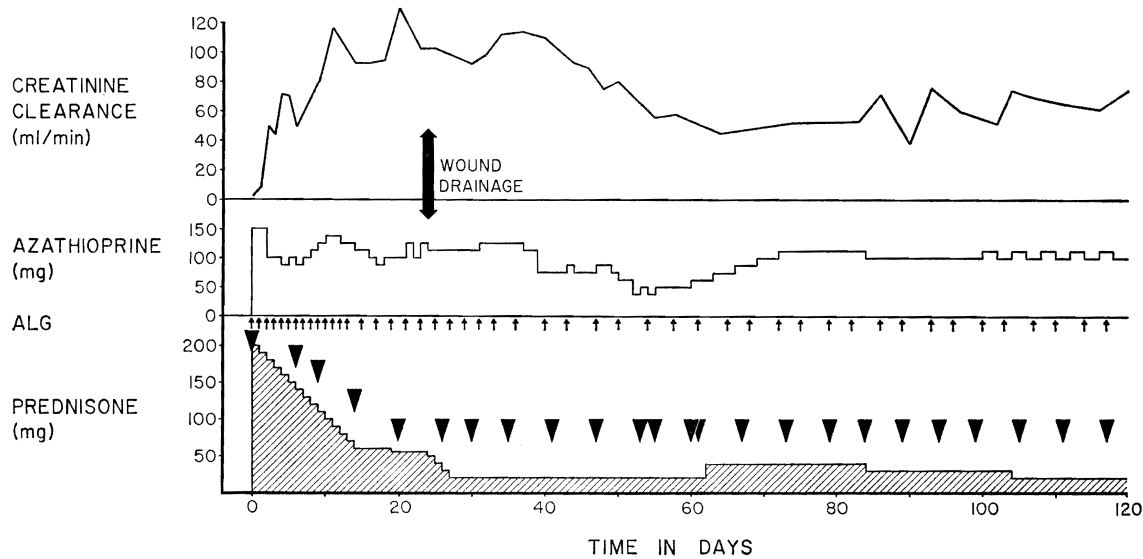


FIG. 1. An example of the use of intermittent intravenously administered prednisolone in a human recipient of a cadaveric renal homograft. One day after transplantation, it was learned that the donor had bacteremia, and it was assumed that the operative wound would become infected. Rapid steroid withdrawal was combined with intermittent intravenous infusions of 1 gram of prednisolone, triangles. The wound was opened widely at three weeks and subsequently healed completely. Eventually, an adjustment was made in the orally administered prednisone maintenance dosage. Small arrows represent intramuscular injections of horse antilymphocyte globulin.

Plasma 17-hydroxycorticosteroids were measured by Wallace and his colleague's modification of the method of Silber and Porter. Urinary Porter-Silber chromogens were measured by the same technique before and after glucuronidase hydrolysis. A Coulter counter was used for white blood counts and hematocrit values. The differential counts were carried out from 500 leukocytes in order to receive more exact absolute lymphocyte counts.

A series of intradermal skin tests was carried out in five of the healthy volunteers to determine the effect of a large dose of prednisolone upon delayed hypersensitivity to mumps, *Candida*, vaccinia, and first strength purified protein derivative tuberculin. The volunteers were known to have been effectively vaccinated against smallpox. They also thought themselves to have positive reactions to the mumps, *Candida*, and tuberculin antigens. However, to avoid inadvertent sensitization, control studies to establish the existence of these hypersensitivity reactions were deferred, and the first intra-

dermal injections of the antigens were made two hours after the prednisolone infusion. Twenty-four and 48 hours later, second and third intradermal doses were repeated. The skin reactions were classified from negative to 4+ according to the size of the induration and the area of erythema (Table I). Two to four weeks after completion of the prednisolone experiment, the mumps, *Candida*, and tuberculin injections were repeated and the results accepted as controls for the previously performed skin tests. Control tests with vaccinia were omitted because delayed hypersensitivity was regularly evident even under prednisolone treatment.

RESULTS

Animal Studies

The rejection times were approximately the same in the New Zealand and California rabbits. The mean value of graft survival in the eight untreated rabbits of group 1 was 8.5 ± 1.8 (standard deviation) days. The rabbits of group 2 which were treated with 0.1 milligram per kilogram per day of methyl-

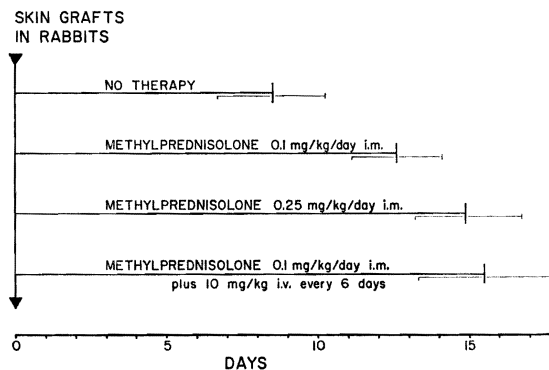


FIG. 2. Rejection times and standard deviations after transplantation of full thickness skin grafts to treated and untreated rabbits.

prednisolone had a prolongation of graft survival to 12.6 ± 1.5 (S.D.) days ($p < 0.001$). With two and one-half times this daily dose of methylprednisolone, rejection in group 3 was further delayed (Fig. 2) to 14.8 ± 1.6 (S.D.) days ($p < 0.001$). The fourth group of rabbits given daily low doses of methylprednisolone supplemented with large single

injections every six days had the longest graft survival, averaging 15.5 ± 2.3 (S.D.) days (Fig. 2). The delay of rejection in the rabbits of group 4 was statistically significant ($p < 0.001$) in respect to groups 1 and 2 but not to group 3.

Human Studies

Subjective reactions.—Of the 16 subjects given intravenously administered prednisolone, eight complained of pruritus which was either generalized or localized to the perineal region. Four physicians among the six normal volunteers also complained of a peculiar taste which was described either as metallic or like phenol. One gram of prednisolone sodium phosphate (Hydeltrasol®) contains 1.25 grams of nicotinamide, 250 milligrams of phenol, 25 milligrams of edetate disodium, and 50 milligrams of disodium bisulfite. For this reason, the six normal volunteers underwent placebo infusions containing the additives and diluent but no

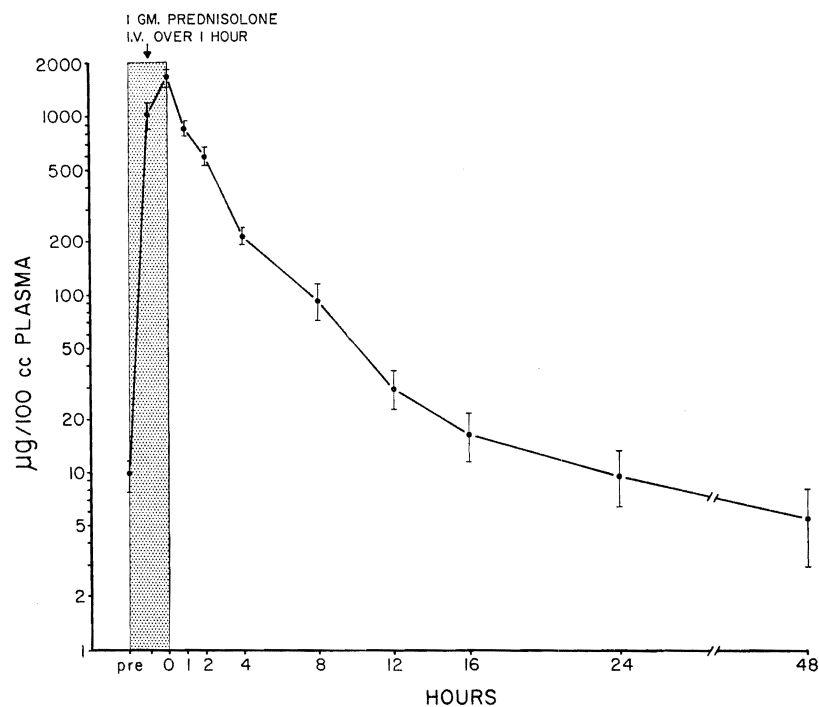


FIG. 3. Micrograms of Porter-Silber chromogens per 100 milliliters of plasma in ten patients and six normal volunteers who were given an intravenous infusion of 1 gram of prednisolone. Vertical brackets are standard errors of the mean. The Porter-Silber chromogen concentrations are expressions of the total plasma steroids.

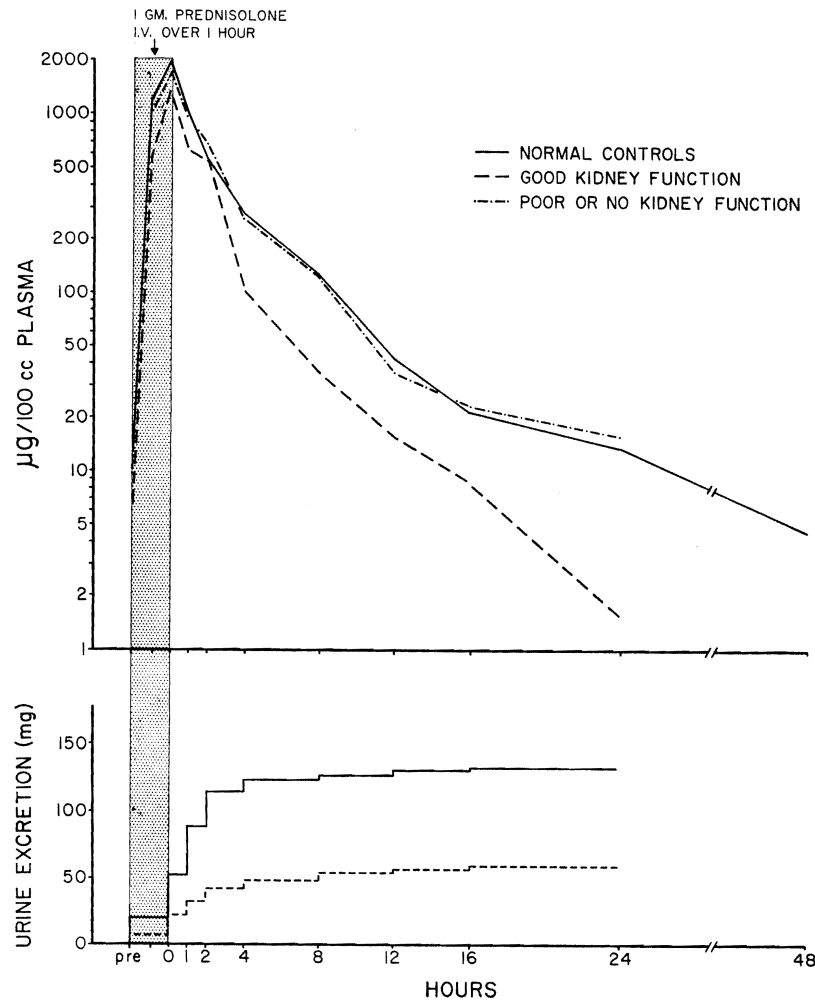


FIG. 4. Comparison of the clearance of prednisolone in six normal, healthy volunteers; five homograft recipients with good kidney function; and five patients with essentially no kidney function. The plasma steroid concentration measured as Porter-Silber chromogens followed about the same curve in all three groups of subjects. The urinary excretion of steroids in the two groups with renal function is expressed in total milligrams of prednisolone.

prednisolone. The identical symptoms of itching and taste aberrations were reproduced.

Prednisolone plasma levels.—The plasma concentrations of Porter-Silber chromogens for the six normal volunteers as well as the ten transplant patients are represented in Figure 3. With the infusion, the plasma levels rose from the rather low pre-existing values to peaks that were usually over 1,500 micrograms per cent. Within a day, or frequently even earlier, the concentrations had

generally returned to the control status. The rapid drop of the plasma levels indicated exponential disappearance of the steroid with an initial biologic half life of only 60 to 90 minutes.

The pattern of changing steroid plasma concentrations was somewhat variable between the six normal subjects, the five transplant recipients with adequate renal function, and the five patients with essentially no renal function (Fig. 4). However, the general events of steroid disappearance from

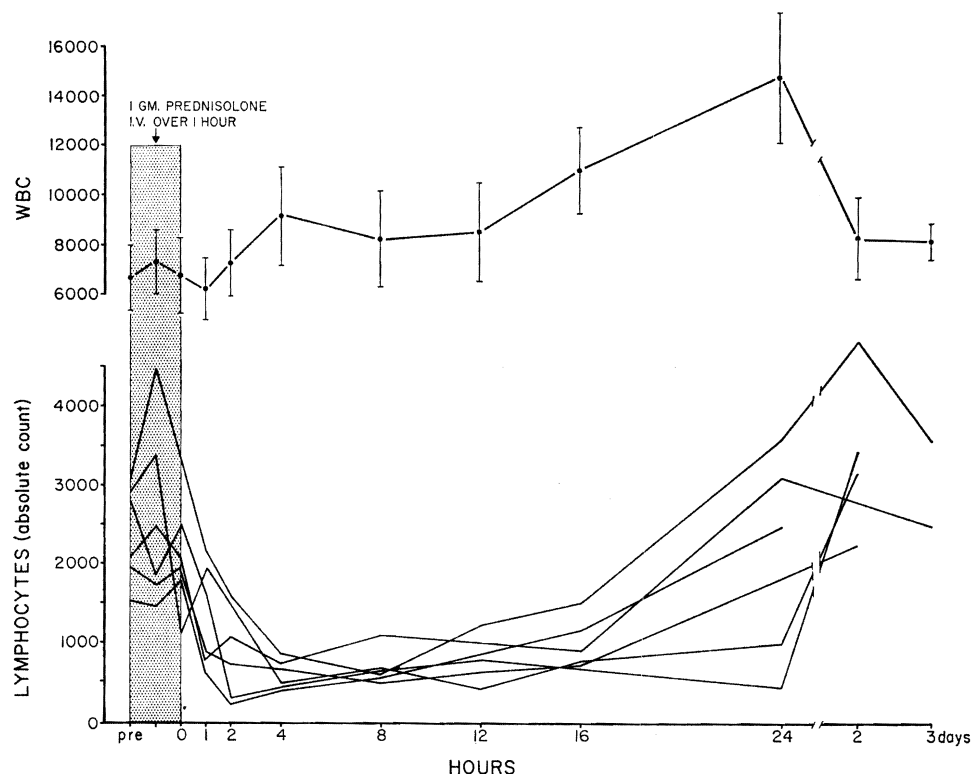


FIG. 5. White blood cell and lymphocyte counts in six normal, healthy volunteers who received prednisolone infusions. The vertical brackets in the white blood cell curve represent standard deviations of the mean. The lymphocyte counts are shown individually.

the plasma were the same in these three subgroups and did not seem to be critically influenced by the presence or absence of renal function (Fig. 4). In the six normal volunteers, it was of interest that the plasma steroid levels fell to the subnormal concentration of 5.5 micrograms per cent after 48 hours, suggesting pituitary-adrenal suppression (Fig. 4).

Urinary steroid excretion.—Porter-Silber chromogens in the urine were quantitated before and after glucuronidase hydrolysis. Without hydrolysis, the results reflected only free steroids, whereas after hydrolysis, the results were a measure of total urinary steroids. The latter values which were 50 to 100 per cent higher than those of free steroids are summarized in Figure 4.

The six normal volunteers had the maximum urinary excretion during and just after the prednisolone infusion, with a peak

of 33 milligrams per hour. However, the total amount of Porter-Silber chromogens recovered in the first day was calculated to represent only 131 milligrams of prednisolone or 13.1 per cent of the injected dose. The five patients in whom renal homografts were functioning adequately had a similar urinary excretion pattern (Fig. 4) but with a total output of only 57 milligrams or 5.7 per cent of the original injection.

Other urinary findings.—Between 4 and 16 hours after the prednisolone infusion, the urine of the six volunteers which had been refrigerated at 4 degrees C. was found to contain a precipitate. The urine was acid with a pH of 5 to 6, and the precipitates were identified as amorphous urates. In three of the six volunteers, a positive glucose reaction developed transiently in the urine two to 12 hours after the infusion.

Hematologic studies.—The white blood

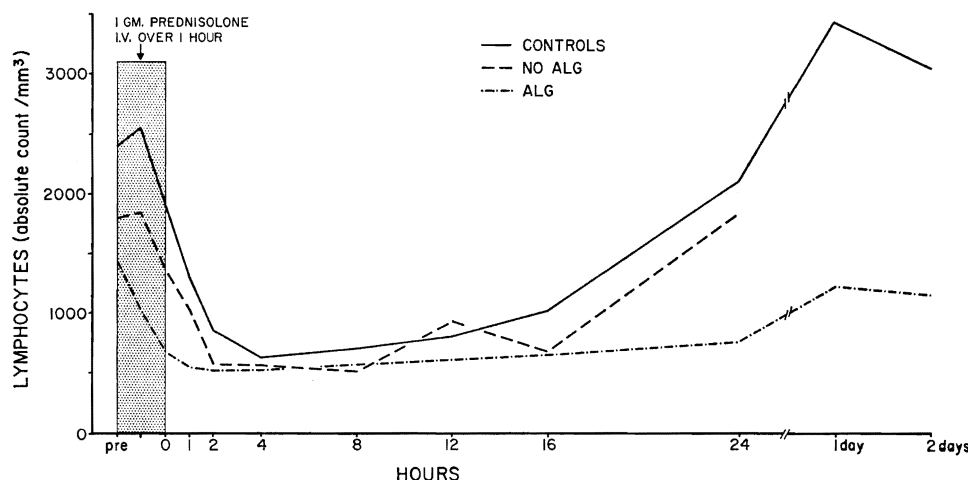


FIG. 6. Average lymphocyte counts in six normal volunteers, three patients who were not receiving heterologous antilymphocyte globulin, and seven patients who were on concomitant therapy with the immune globulin. Note that recovery of the lymphocytes seemed to be less prompt in the group under treatment with antilymphocyte globulin.

counts in each of the six healthy volunteers are charted in Figure 5. Before the infusion was begun, the average count was 6,680 per cubic millimeter. At four, 16, and 24 hours, leukocytosis was observed which at the last two times was statistically significant ($p < 0.05$). At two and three days, the counts had returned to normal range. The absolute lymphocyte counts prior to the infusion of prednisolone were $2,390 \pm 620$ (S.D.) per cubic millimeter. Two hours after completion of the steroid injection, highly significant depressions of the lymphocyte counts occurred (Fig. 5) to a nadir of 630 ± 180 (S.D.) per cubic millimeter at four hours. The lymphopenia persisted for most of the first day, and then the lymphocyte counts rose to normal or supernormal values.

In principle, the results in the ten patients were similar to those in the normal volunteers. However, the seven recipients who were also receiving azathioprine and antilymphocyte globulin had lymphopenia which developed more quickly and was more prolonged. In these patients, the maximum lymphocyte depression to 520 ± 350 (S.D.) per cubic millimeter occurred at two hours after the administration of prednisolone and remained significant for most of the first day. By 48 hours, there was no re-

bound. The three not being given antilymphocyte globulin had results that were intermediate between the healthy volunteers and globulin-treated recipients (Fig. 6).

Hematocrit changes were not noted in either the volunteers or the patients. In both groups, eosinophiles disappeared for two to three days after a prednisolone infusion. The monocyte counts were also depressed, but this was not statistically significant at any time. Dramatic morphologic alterations were not seen in the cells studied on blood smear.

Delayed hypersensitivity skin tests.—Data on the results with skin testing are shown in Table I. When either mumps or *Candida* antigen were given two hours after the prednisolone infusion, evidence of a significant suppression of response always occurred at the time of the one day reading and often even at the two and three day readings. If these antigens were reinjected 24 or 48 hours after the steroid infusion, the delayed hypersensitivity reaction expressed itself but still subnormally in several patients (Table I). The same kinds of observations were made in two of the volunteers who were tested with tuberculin (Table I). The steroid infusions mitigated the skin reaction to vaccinia antigen only slightly (Table I).

DISCUSSION

Adrenocorticoid steroids were the first immunosuppressive agents to be systematically evaluated. During the several years after the first reports by Billingham, and his colleagues and by Morgan, there were several confirmatory descriptions of the ability of cortisone acetate to significantly delay the rejection of first set skin grafts in rodents; the same effect was seen in chickens by Cannon and Longmire. Furthermore, it was demonstrated by Krohn, as early as 1954, that cortisone acetate could abolish a pre-existing state of sensitivity induced by full thickness skin homografts in rabbits that were rejected in about nine days. Thirty-seven to 60 days after the primary exposure, regrafting was carried out with skin from the same donor. An accelerated rejection was avoided if subcutaneous steroid therapy, 10 milligrams per day, had been instituted several days in advance of the second operation; in fact, the second transplants often survived longer than had been the case with the untreated first ones.

The results with prednisolone and methylprednisolone herein reported were not essentially different than the pioneering studies with cortisone acetate. There was a limited but definitive prolongation of survival of first skin grafts in rabbits. Moreover, the human studies showed that the expression of pre-existing hypersensitivity could be blunted or, in some cases, prevented altogether.

The most important new and practical questions asked in this investigation were, first, if intermittent large doses of steroids would significantly potentiate the immunosuppression of smaller daily quantities and, second, if such a potentiating effect were simply due to a chronically elevated blood level of the prednisolone or methylprednisolone that lingered on long after the intermittent infusions. The answer to the first question was yes. In rabbits, the viability of full thickness skin grafts was prolonged by adding the large intravenous injections every six days to the maintenance therapy at 0.1

milligram per kilogram per day. The results were slightly better than those obtained by simply increasing the day-to-day doses by two and one-half times.

Information about the second question was equally decisive. Improvement in graft survival with the intermittent large doses was not explainable simply by a persistent elevation in the plasma steroids. In both the patients and healthy volunteers, 1 gram of prednisolone was cleared from the blood stream within a day or frequently sooner. Similar rapid disappearance rates of cortisol have been described in human beings by Peterson. With prednisolone, the exact mechanism of clearance is not known, although it was clear from the observations that renal excretion was not essential for the elimination. In patients with well functioning renal homografts and in normal volunteers, the prednisolone recovered from the urine was only 5.7 and 13.1 per cent, respectively, the exact percentages being a rough correlate of the creatinine clearance. Moreover, in anephric patients in whom a renal pathway of degradation did not exist, the prednisolone disappeared from the blood stream at a perfectly normal rate.

The demonstration of such rapid disappearance of the plasma steroids carried the implication that the immunosuppressive effect of a single high dose outlasted the degradation, at least for some hours and perhaps for several days. Such an assumption was consistent with the results in the animal experiments as well as with the observation in the human beings who failed to respond normally to an antigenic challenge given 24 hours after the prednisolone infusion.

It is probable that at least part of the explanation for the protracted effect from a single steroid dose was the lymphopenia which occurred promptly and invariably and which usually lasted for most of a day. The articles by White, Bjorneboe, Gabrielson, and by Nelson and their associates could be cited in support of this concept since they described lymphoid depletion after

steroid therapy. There was no direct evidence in our experiments that the steroid infusions selectively affected the small, long-lived, antigen-sensitive lymphocytes of thymic origin as opposed to the large, short-lived lymphocytes which have been said by Dougherty, Gabrielson, Elves and by Batra and their associates to be cortisone-resistant. However, the experiments were not specifically designed to obtain this kind of information.

Whatever the reasons for the effectiveness of intermittent high steroid doses, the practice would appear to have merit in immunosuppressive regimens. In our clinical experience, the infusions have been extremely well tolerated, and in the rabbit experiments, they have seemed to permit mitigation of homograft rejection with much smaller and safer daily quantities than would otherwise have been anticipated. With the intermittent technique, a number of patients with major infectious complications have been quickly weaned from unacceptably large maintenance doses of prednisone but with retention of good homograft function.

SUMMARY

Full thickness skin grafts were exchanged between 16 New Zealand and 16 California rabbits. Intramuscularly administered methylprednisolone prolonged the average survival of the grafts from 8.5 to 12.6 days when given in doses of 0.1 milligram per kilogram per day and to 14.8 days when given in doses of 0.25 milligram per kilogram per day. When single injections of 10 milligrams per kilogram were added intravenously every six days to the lesser of the foregoing maintenance doses, the graft survived an average of 15.5 days.

Six normal volunteers and ten patients with variable renal function were given an intravenous infusion of 1 gram of prednisolone over 60 minutes. The plasma steroid levels rose to prodigious heights, but then returned to normal within 12 to 24 hours, by an elimination process that was not de-

pendent on renal function. Lymphopenia was produced along with variable changes in the total white blood count. The expression of delayed hypersensitivity to mumps, Candida, tuberculin, and vaccinia antigens was either prevented or weakened for at least the first day after the steroid injection.

These experiments support the validity of a clinical practice that has developed more or less empirically in the treatment of homograft recipients, namely the intermittent intravenous administration of large quantities of steroids as a supplement to low maintenance doses. The additional immunosuppressive effect thereby achieved cannot be explained simply by a persistence of plasma steroid levels between the special infusions since the disappearance of the intravenously administered prednisolone occurs too rapidly.

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